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CHAPTER 1

Physiological and molecular aspects of growth, non-growth, culturability and viability in bacteria

M. R. Barer

INTRODUCTION

Infection requires growth of pathogens in host tissues or on host epithelia. Cessation of growth is generally correlated with control of infection. Clinically latent infections may reflect microbial growth balanced by host control mechanisms such that the interaction remains below the threshold of detection. Alternatively, the pathogen may have genuinely ceased growth and survive in some form of stasis. In most cases we cannot distinguish between these possibilities. However, there have been important recent advances in our understanding of bacterial populations in which net growth cannot be detected and in recognising the limitations of *in vitro* culture as a means of determining the presence and viability of bacteria. These advances present new opportunities to study the role of non-growing and dormant bacteria in infection and to consider the degree to which culture-based methods may give a false impression of the absence of pathogens during infection, clinical latency and treatment.

The progress of molecular methods in microbiology challenges us to determine the molecular basis of growth and its regulation and to develop such methods to detect growth and viability. In the present context, the long-term aim must be to recognise growth states of microbial populations in the human host.

BACTERIAL GROWTH

Growth involves the accumulation of biomass and may include genomic replication, cell division and an increase in the number of propagules of the organism concerned. For most bacteria it is generally held that, after division,

a newly formed cell placed in an environment favourable to growth will double its mass then divide to form two equal-sized progeny via binary fission. This process has been subjected to detailed analysis and is discussed from a highly selective viewpoint here. For more comprehensive and introductory discussions, the reader is referred to recent reviews (35, 55, 57, 66, 82, 104).

Our current understanding of bacterial growth derives overwhelmingly from studying selected organisms in broth cultures. Liquid cultures are convenient; most variables can be precisely controlled, and the scale can be adjusted to provide sufficient biomass for almost any form of analysis. In achieving reproducible results between laboratories, the development of chemically defined media, consistent inocula and the recognition of growth states that can be detected by sequential optical density or turbidity measurements have provided a platform for further development. The widely accepted terminology of lag, exponential (or log) and stationary phases of growth in batch culture provides essential physiological points of reference and these are often applied, with scant justification, to bacterial cells and populations outside the highly defined laboratory environments indicated.

A detailed analysis of the energetics and stoichiometry of bacterial growth has been made possible by analysing bacterial populations growing at constant rates in chemostat or turbidostat cultures (35, 57, 93). These systems provide a relatively reproducible gold standard in which a state referred to as "balanced exponential growth" can be achieved for extended times. The resultant population of cells is generally believed to be uniform and growing at similar rates. Thus it is considered legitimate that analyses of cells in balanced exponential growth can be divided equally amongst all the cells present in the sample to yield estimates of content or activity per cell present.

An important alternative approach has been to start by considering the bacterial cell cycle, which starts with the birth of a cell by binary fission of a parental cell and ends with the division of the new cell. This kind of work draws substantially on our understanding of the eukaryotic cell cycle, where the biochemical and physiological events have been separated into distinct phases (G_1, S, G_2, and M with or without G_0), and has been pursued using techniques that provide large populations of cells that are all at the same stage of the cycle. While some controversy continues, it is generally thought that events that are considered critical for progression through the cell cycle in eukaryotes (e.g., initiation and termination of DNA synthesis) are not similarly regulated in bacteria. Rather, the short-term fate of a cell is determined by the rate at which it accumulates biomass and by the particular size:growth

rate ratios at which division is initiated (18). Recently, however, Walker and colleagues (120) have suggested that the *umuDC* component of the bacterial SOS response functions in a manner analogous to the eukaryotic S phase checkpoint. The analogy is complicated by the fact that rapidly growing bacteria initiate new rounds of chromosome synthesis before the last has finished. The authors also point out that the associated checkpoint and DNA repair systems are well suited to dealing with DNA damage accumulated during stationary phase at the time of re-entry into the growth cycle (92).

Most biochemical knowledge obtained with these methods refers to large cell populations ($>10^7$) of readily culturable bacteria in exponential growth phase. Here, we are primarily concerned with the behaviour of pathogens during infections. Not only will these organisms rarely be in a simple suspension phase but also it seems most unlikely that the environment will be conducive to unimpeded exponential growth. Evidently, the degree to which most of our knowledge of bacterial growth is applicable to the environments that primarily concern us must be limited.

Laboratory studies on bacterial growth have also provided limited information regarding growth in colonies on or in solidified laboratory media (74, 125) and in biofilms (37, 75). While information on the growth of bacteria in colonies and in broth may be valuable in designing isolation and culture media for medically important bacteria (35), growth in biofilms is probably a principal mode of bacterial propagation in natural communities. In infections involving fluid-filled spaces (e.g., cystitis) it is plausible that the growth phases recognised in broth culture may be applicable and the relevance of biofilm growth to colonization of intravascular devices also seems certain. However, beyond these examples, assignment of *in vitro*-defined growth phases to pathogens at various stages in infection is largely speculative.

Molecular Information Related to Bacterial Growth

Studies on carefully defined broth cultures remain the principal reliable source of information on the molecular basis of bacterial growth. As key genes involved in growth and its regulation have been identified through recent pre- and post-genomic studies, the possibility of determining the importance of these genes to infection through deletion, over-expression and reporter studies has been extensively exploited. In the context of infection, it is conspicuous that technologies applied to detection of genes essential for growth *in vivo*, such as signature tagged mutagenesis, have often detected genes that appear integral to growth and metabolism (as opposed to classical

aspects of virulence such as invasion and toxicity) as essential for *in vivo* survival (e.g., 63, 94).

A somewhat arbitrary selection of genes whose expression has been related to growth in various ways is reviewed below. Ultimately it should be possible to recognise all the genes that are required for growth in specific environments. It seems likely that these will fall into two categories: those required in all circumstances and those required only for special environments.

Ribosomal RNA

A single *E. coli* chromosome generally carries seven copies of the genes encoding ribosomal RNA. In contrast, the *Mycobacterium tuberculosis* chromosome encodes only one copy. Given the greater than tenfold difference in minimum doubling times between these organisms (0.3 h vs. 6 h), it seems likely that this is no accident. The 16S, 23S and 5S genes (and some tRNA genes) are located in tandem and are initially transcribed into RNA as a single molecule, which therefore includes the so-called intergenic transcribed sequences (ITSs). The transcript is then processed into the recognised subunit components, and these combine with ribosomal protein to form functional ribosomes. Aside from the central role now occupied by the 16S molecule and the ITSs in the classifications of *Bacteria* and *Archaea*, the rate at which these genes are transcribed and the 16SrRNA content of bacterial cells has been directly correlated with bacterial growth rates *in vitro* (14, 20). Analysis of these genes and their products in samples therefore presents opportunities to both identify and make some inferences about the protein synthetic capacity and growth rate(s) of the organisms present.

Chromosome Replication

Chromosome replication requires more time to complete than the time available between cell divisions during rapid growth of *E. coli*. The organism circumvents the potential problem of producing cells with less than a single complete genome by initiating rounds of chromosome replication at intervals compatible with the cell replication rate. Initiation always starts at the same locus (*oriC*) and proceeds bi-directionally to the terminus region (76). One consequence of this is that cells in rapidly growing populations contain more than one chromosome replication fork in progress, and the largest cells present (i.e., those close to fission) have a chromosomal DNA content in excess of two copies of the complete genome. The mechanism by which the interval between initiating rounds of chromosomal replication is regulated is not understood, but several gene products are known to be essential. Amongst these the DnaA protein, a DNA-, ATP- and ADP-binding

protein, has been most extensively studied and appears to play a central role in assembly of the initiation complex (76). A further consequence of the pattern of replication is suppression of transcription of specific genes as the replication fork passes through. This leads to apparent cell-cycle-related gene regulation in synchronised cultures (129).

Cell Division

Understanding of the molecular basis of bacterial cell division has advanced dramatically over recent years. Progress has been fuelled by development of immunocytochemical techniques for bacteriology and by the use of translational reporter fusions with the green fluorescent protein. These developments have enabled localisation of key molecules that determine the site and process of cell division. Amongst these the tubulin-like molecule FtsZ has been extensively studied. Around 10,000 molecules of this key protein are present in each *E. coli* cell and, like its eukaryotic counterpart, it is present in both soluble and polymerised forms. Location of FtsZ polymers in ring structures indicates the site of prospective septum formation, and using *ftsZ::gfp* translational fusions, it has been possible to observe, in real time, the formation and subsequent contraction of the FtsZ ring in parallel with septum formation and cell division (67, 118). Although FtsZ possesses GTPase activity, it is not known whether it provides the physical force required for septation and fission. Inhibition of FtsZ polymerisation by SulA (a protein produced as part of the SOS response) in growing cells leads to filamentation, thereby illustrating the key role of FtsZ in fission. All bacteria so far studied possess FtsZ homologues, and the relative abundance of the molecule makes it an attractive target for study in clinical samples. The presence of FtsZ rings indicates active cell division, and in *Bacillus subtilis*, asymmetric positioning of the ring indicates the onset of sporulation (64).

Global Regulatory Proteins

These molecules direct differential gene expression by binding either to DNA or to components of the transcription/translation apparatus. Their own levels of expression and activity are modulated by a variety of internal and external stimuli. It would be impractical to discuss even a small minority of these molecules here, but the levels and/or activities of some prominent examples in relation to bacterial growth are outlined in Table 1.1. A full discussion of these molecules is in Chapter 2.

A discussion of the complex regulatory hierarchy and network that are emerging from the study of these proteins and their cognate regulons is beyond the scope of this chapter. The painstaking process of analysing their

Table 1.1. *Regulatory proteins associated with different aspects of growth in bacteria*

Category	Protein	Gene(s)	Some relationships to growth	Function
Regulation via DNA topology[1]	H-NS	*osmZ*	Levels in constant ratio to DNA content during growth. Depressed in stationary phase	Histone-like DNA binding protein that represses transcription of multiple genes
	LRP	*lrp*	Repressed by growth in rich medium	Selective repression and activation of genes appropriate to available nutrient sources
	IHF	*ihfA (himA)* *ihfB (himD)*	Induction on entry into stationary phase; expression dependent on ppGpp	Interaction with DNA induces 180° bend enabling long-range interactions
SOS response[2]	RecA	*recA*	Acitvation of RecA by DNA damage induces cleavage of LexA and de-repression of SOS genes and arrest of cell division.	RecA controlled genes effect DNA repair and maintain λ-like phage lysogeny.
	LexA	*lexA*		
Alternate σ factors[3] (required for transcription)	σ^{70}	*rpoD*	σ^{S} levels increase on entry into stationary phase and on sudden growth arrest. Alternate σ factors appear to compete with σ^{70} for binding to a limited amount of core RNA polymerase. Promoter specificity is modulated by alternate σ factor binding	Main RNA polymerase σ subunit
	σ^{38} (σ^{S})	*rpoS*		Stationary phase and stress induced
	σ^{32}	*rpoH*		Heat shock induced
	σ^{24} (σ^{E})	*rpoE*		Induced by extreme heat shock and regulates extracellular proteins
	σ^{28} (σ^{F})	*fliA*		Flagellar gene regulation
	σ^{54}	*rpoN(glnF)*		

			in combination with many other factors (e.g., H–NS, LRP, IHF & ppGpp). In *B. subtilis* a succession of alternate σ factors directs the programme of gene expression in sporulation.	Control of nitrogen metabolism
Universal stress response proteins[4]	UspA	*uspA*	Induced in late exponential phase and by all known stress responses (σ^{70} and ppGpp dependent)	Regulation via phosphorylation of target proteins?
	UspB	*uspB*	Induced during transition phase (σ^{38} and ppGpp dependent)	Unknown
General metabolic regulators[5]	CRP	*crp*	Low glucose (e.g., on entry into stationary phase) leads to increased cAMP levels.	CRP–cAMP complexes activate or repress specific genes.
	FNR	*fnr (nirA)*	FNR senses oxygen (anaerobic/aerobic growth)	Reduced FNR activates or represses specific genes related to anaerobiosis.
	ArcA	*arcA*	ArcA phosphorylated by cell membrane–associated ArcB quinone-responsive sensor kinase	Regulates catabolism of metabolic reserves

Note: Information presented predominantly relates to studies on *E. coli*. Abbreviations: IHF – integration host factor; USP – universal stress protein; CRP – cAMP receptor protein.

General sources: 78, 82, 83, 106. *Specific sources:* [1](3, 13, 24, 69); [2](120); [3](27, 29, 40, 64, 98, 109); [4](30, 33, 88); [5](11, 31, 34, 52, 62, 87, 105, 107).

respective roles is really only in its early stages and doubtless there are many regulators yet to be recognised and more functions to be defined. The relationships of these molecules to growth suggested in Table 1.1 emerge essentially from studies on samples from populations in specific growth phases during growth in defined media. With a few notable exceptions, information (e.g., 68) on the expression of regulatory proteins in contexts relevant to infection is very limited.

It should not be forgotten that there are many other classes of molecule that regulate bacterial phenotype. The underlying point here is that phenotype and growth state cannot necessarily be inferred from the detection of selective mRNA profiles. At the macromolecular level selective proteases (38) and antisense RNAs (121) have received much attention. Small molecules such as cyclic AMP (cAMP) (11) and guanosine tetraphosphate (ppGpp) (17, 43) are also recognised to have important regulatory roles. Many gene products affect their intracellular levels, and they have plieotropic allosteric effects on their respective binding proteins. The role of ppGpp, the key product of the stringent response, deserves special mention here since, by binding to the B subunit of DNA-dependent RNA polymerase, it provides another means of directing selective gene expression. The stringent response is stimulated by amino acid starvation and is generally associated with growth arrest (17).

In this selective survey the obviously important areas of energy metabolism, cell envelope biosynthesis and assembly and the so-called housekeeping genes have largely been ignored. However, the process of relating the expression of genes to bacterial growth could be extended to cover the entire genome and this can serve little function until we have an adequate interpretive framework. The global approaches offered by proteomics and arrays provide realistic prospects that this will be achieved.

GROWTH AND STASIS

The growth phases of bacteria in batch culture have been reviewed extensively elsewhere (35, 82, 93). Here the focus will be on individual cells and the populations they comprise. The aim is to introduce a framework within which cells in physiological states of particular significance to infection can be recognised and to cross-reference this to the classical growth phases.

Figure 1.1 presents a diagram outlining the various physiological states that can be recognised in relation to the growth of bacteria. Laboratory cultures can be observed at the population or cellular level and a comparison between these is attempted in Fig. 1.1. A central dichotomy is suggested between cells growing or committed to grow and those in some form of non-growing

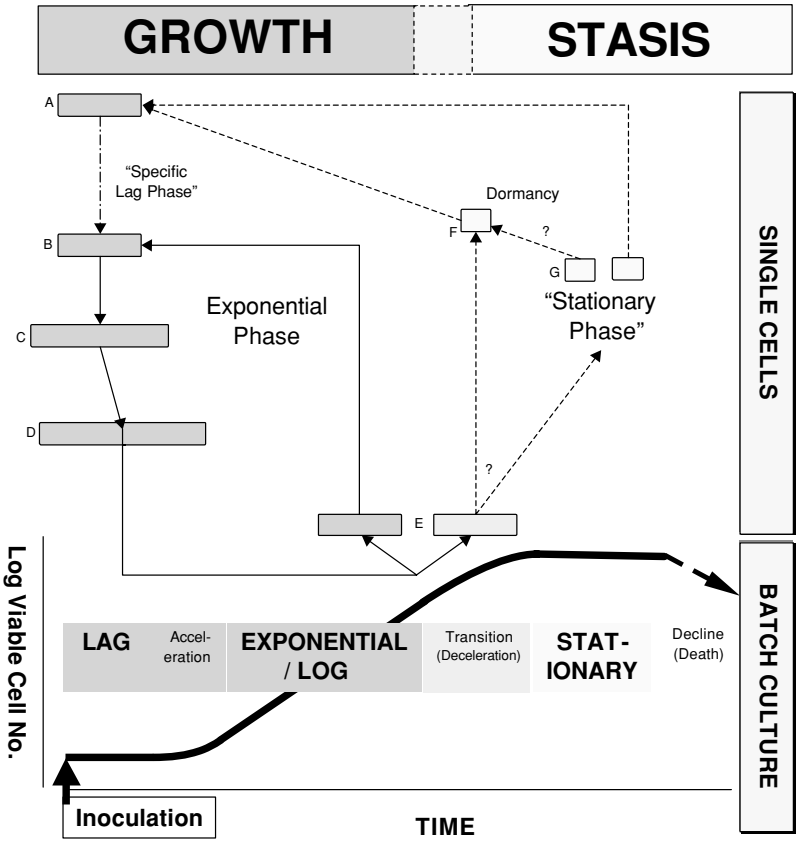

Figure 1.1. Diagram comparing recognised states of growth and stasis in single cells and populations of bacteria. Point A identifies a cell committed to growth and replication; B, the initiation of growth; C, accumulated biomass below that required to initiate septation; D, septation prior to fission. E represents the point at which cells are notionally committed either to continued growth (equivalent to B) or to stasis. The conditions required for commitment to either pathway are only recognised at the population level. G represents cells that are not accumulating biomass, and F represents cells that may be formally described as dormant.

state (stasis). All the states possess potential for further growth and replication, and the cells concerned should therefore be considered viable.

Exponential Phase

In state A, a hypothetical cell, committed to growth and with appropriate resources available but not yet detectably growing, is envisaged. This cell may

be adapting to a new environment or recovering from injury. Eventually the cell achieves state B where its phenotype is adapted to commence growth in its current environment and is seen as equivalent to the product of division in a growing culture. This cell grows as indicated through state C, where septum formation is initiated, to state D, where the septum is completed and fission is in progress. The separation of the progeny (E) into growing and static cells is arbitrary and serves only to illustrate alternate pathways. If conditions were conducive to continued growth then both progeny would be expected to continue in exponential growth.

The period between A and B is tentatively referred to here as the "specific lag phase." In operational terms, the lag phase is measured as the interval between inoculation and the onset of detectable growth and can include an initial period of cell death and growth below the limit of detection. This period may include the times indicated between G and A or F and A, i.e., the time taken for non-growing cells to adapt and become committed to growth (see below).

An enormous amount of knowledge has been gained about populations dominated by cells in the exponential phase of the growth cycle indicated in Fig. 1.1. The rapidity of biomass accumulation is potentially breathtaking with doubling times of less than 30 minutes readily achievable by many pathogens that cause acute infection. It seems likely that such growth rates could underpin the rapid development of some infective conditions. The gradient of the exponential phase is dependent on the environmental conditions and the organism. *In vitro,* unrestricted (exponential) growth in chemostat cultures is amenable to quite sophisticated mathematical analysis (e.g., 35, 57, 93).

Bacterial physiological responses to environmental changes have mainly been studied using exponential phase cells. Where these changes are potentially lethal, the responses are referred to as stress responses. The genetic basis for the phenotypic changes elicited by environmental change has been studied extensively, initially by mutational and reporter analysis and at the proteomic level (10, 32, 40, 41, 115, 127). Latterly, genomic and subgenomic arrays have afforded an attractive approach to studying these adaptive responses at a global transcriptional level (119). Depending on the nature of the environmental change or stress, the changes in gene expression elicited may involve between tens and hundreds of different genes. Where the change is not stressful (as defined above) it appears that growth is substantially slowed down or arrested and resumes after the adaptation is complete. In some cases, notably where nutrient depletion precludes further growth, changes in the pattern of gene expression are not confined to a single shift but rather a sequential programme of change is entered into (54). Where this results in a defined morphological adaptive change, such as in sporulation, it is referred

to as differentiation (28). In contrast, if the change is stressful (e.g., a substantial pH change or temperature increase) a proportion of the population is killed. The adaptive response in the survivors makes them at least temporarily more resistant (a higher proportion of survivors) to further similar stress.

Stress-responsive genes may be activated by one or more stimuli and in some cases multiple stimuli. Three examples of such multiply responsive genes, *rpoS*, *uspA* and *uspB*, are cited in Table 1.1. It is conspicuous that all three are upregulated in stationary phase. This lends weight to the view that the arrest of net growth in batch culture referred to as stationary phase is itself a form of stress response related to either nutrient depletion or accumulation of toxic metabolites. Several stress responses confer cross-protection against other stresses (e.g., pH and heat) and stationary phase populations are generally more resistant to stress than exponential phase populations (2, 40). (See Chapter 2 for a full discussion of survival of stress.)

Stationary Phase

Although the classical view of stationary phase is that it reflects growth arrest associated with nutrient exhaustion, recent work has raised the possibility that cessation of growth may sometimes be "elective." Indeed there is evidence that bacterial populations have not exhausted all the nutrients they could grow on when they enter stationary phase (7) and that they may produce specific autocrine signals that tell cells not to grow (59). The possible roles for cell-to-cell communication in regulating growth are discussed further below.

Returning to Fig. 1.1, some of the reasons why cells may enter the stasis section of the cycle have been identified. Apart from the ability to reproduce the phenomenon of the stationary phase in batch cultures and to alter the kinetics of its onset by the composition of the growth medium, little is known about how the transition from exponential to stationary phase is regulated. A summary of the recognised influences is given in Fig. 1.2. It is certainly clear that genes such as *uspA*, *uspB* and *rpoS* are upregulated prior to or coincident with the onset of the cessation of net detectable growth (cf. Table 1.1), and it seems likely that they play significant regulatory roles. However, it must be emphasised that, even *in vitro*, it is very difficult to achieve uniform bacterial populations and the standard methods of analysis reflect only gross changes in biomass and dominant biochemical properties. It is therefore entirely possible that, in batch culture, cell populations characterized as being in exponential or stationary phase in fact comprise mixtures of cells in all of the states identified in Fig. 1.1 but in different proportions (e.g.,

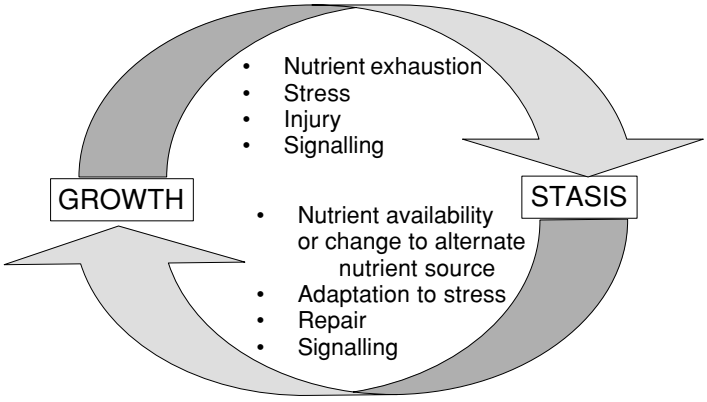

Figure 1.2. Diagram illustrating factors influencing growth and stasis at the population level.

100:1 growing:static in exponential phase; 100:1 static:growing in stationary phase). Only in the chemostat or turbidostat, or after multiple rounds of growth and dilution prior to stationary phase, will the static population be kept to a minimum (but still not eliminated).

An important consequence of this last point is that it is very difficult to attribute specific patterns of gene expression to specific cell populations when the measurements have been performed at the population level. Thus, for example, when we observe changes in the patterns of protein expression on 2-D gels in response to a stress, one cannot be certain that individual changes are happening in all the cells sampled. Indeed it is quite possible that multiple subpopulations are represented. Moreover, the relationships between gene expression and phenotype are rarely determined in such experiments. So, where the stress is lethal to a portion of the population, we cannot even be certain whether the changes are taking place in cells that are going to survive or in those that are going to die. These problems are not insurmountable, but they do show some limitations to the global analytical approach.

From the above it should be apparent that while stationary phase can be recognised as a phenomenon and characterised at the molecular level in batch cultures (56, 70, 113), the notion of a "stationary phase cell" is by no means precise. It is well known that smaller cells with lower ribosomal content dominate stationary phase cultures, but similar statements could be made about chemostat cultures at very low dilution rates. In the case of stationary phase resulting from various nutrient limitations, the smaller cells appear to result from reductive cell divisions (fission without cell growth) (54), but it is not certain that this is always the case. One important feature of

stationary phase cultures is that they are generally more resistant (in terms of maintaining colony-forming unit (CFU) levels to multiple stresses (e.g., removal of C, N or P from the medium, heat shock or antibiotic treatment). This may well relate to upregulation of genes like *rpoS*, the expression of which is associated with several different stress responses. Teleologically this makes sense since, at least notionally, stationary phase implies a relative lack of resources for the bacteria so it would seem prudent to be protected against multiple noxious influences when the capacity to respond is reduced.

Cells that persist in stasis can be considered to be ageing. When stasis is associated with nutrient exhaustion the capacity for turnover and repair in cells is limited. Recent studies have provided evidence that accumulation of oxidative damage to proteins and DNA is a critical aspect of survival under these conditions (25, 26, 87). These and related studies have been drawn together into a framework for understanding metabolic adaptations to stasis in bacteria that could have far-reaching implications for how we approach the control of non-replicating bacterial populations of concern to medicine and public health (85, 86).

Finally, stationary phase cultures are by no means inert. Stress responses can be detected, and at least in the case of acid stress, a response specific to stationary phase can be demonstrated (32). More significantly, at least in terms of Fig. 1.1, stationary phase cultures are not exclusively composed of non-growing cells. Kolter and colleagues (56) have described a phenomenon referred to as "GASP" (growth adapted to stationary phase) in which long-term stationary phase cultures were shown to contain successive growing subpopulations that replicated from CFU levels below the limit of detection to eventually dominate the CFU population. These emergent populations have been specifically attributed to *rpoS* and *lrp* mutations (128, 130). Thus, while the total CFU count remained the same, this concealed dynamic events occurring within the study population.

Dormancy and Sporulation

In contrast to the notional stationary phase cell shown in Fig. 1.1, there are at least some defined examples of dormant cells. Here, the term dormant is used to denote cells in which there has been a reversible shutdown of metabolic activity (47). The bacterial spore provides the clearest example of a dormant bacterial cell. Sporulation is a differentiation pathway involving sequential activation of genes initially in the mother cell and then selectively in the mother and developing spore cell (27, 116). The process provides a

genetic paradigm for differentiation and dormancy in bacteria and has been studied most extensively in *B. subtilis*. In particular, the recognition of the importance of switching between alternate σ factors has provided a useful framework for studying adaptation and differentiation in bacteria. Sporulation leads to the production of highly stress-resistant cells and can be viewed as an extreme form of the adaptations that occur in stationary phase. Indeed, sporulation is initiated in transition phase and by the factors identified in Fig. 1.2. (Note that several peptide signalling factors have been defined in this context (60).) Exactly what decides whether a cell enters stationary phase or dormancy (G or F, Fig. 1.1) is not defined, but it is certainly the case that both spores and vegetative cells are present in stationary phase cultures. This heterogeneous response reinforces the points made above concerning the multiple populations that may be present in bacterial monocultures. (Note that other heterogeneous responses including development of competency and motility also occur during transition phase in *B. subtilis*.)

Somewhat less well defined are the dormant cells of *Micrococcus luteus* described by Kaprelyants, Kell and their colleagues (46, 50). These cells develop slowly after maintenance of stationary phase cultures for several months. In classical terms the decline phase is well established in these cultures since CFU counts have generally fallen by several orders of magnitude by the time the dormant cells can be demonstrated. Although some morphological changes are recognised in the populations containing dormant *M. luteus* cells, Kaprelyants and colleagues explicitly recognise dormant cells by two properties: their substantially reduced capacity to take up rhodamine 123 (a membrane energisation-sensitive fluorescent probe) when compared with exponential or early stationary phase populations and the capacity to be cultured through colony formation or in broth. Through painstakingly careful experiments, these workers were able to demonstrate that dormant cells that could not be cultured by conventional means could nonetheless be resuscitated by exposure to cell-free supernatants from growing *M. luteus* cultures. Subsequently, these supernatants were shown to contain a 17-KD protein that Kaprelyants and colleagues termed resuscitation promoting factor (Rpf) and the cognate gene (*rpf*) was cloned and sequenced (80, 81).

An important distinction must be made between dormancy as exemplified by sporulation and by the *M. luteus* model. Sporulation clearly results from a programme of gene expression that can legitimately be described as differentiation. The *M. luteus* cells meet an operational definition of dormancy (see below) but there is no evidence that they result from a specific genetic programme or that they confer a survival advantage in the way that spores clearly do. Indeed there is evidence that the dormant cells are in fact

"injured" or "moribund" since their permeability properties are demonstrably "repaired" during the resuscitation process (44).

Nonetheless, since there is now nucleotide sequence data from many of the genes involved in sporulation and germination in several genera and from *rpf* in *M. luteus*, the opportunity to determine whether other bacteria encode homologues of these genes arises. Sporulation gene homologues have been found in bacteria that have not been demonstrated to produce spores (e.g., 21). However, caution must be exercised in concluding that these organisms have dormant forms that have not been recognised. Several genes that were first recognised in the context of sporulation are now known to have important functions in vegetative cells.

In contrast, whether or not dormant *M. luteus* cells are comparable to spores, studies on purified Rpf have shown that it has distinctive growth-enhancing properties, notably shortening of the lag phase, that have led its discoverers to describe it as the first "bacterial cytokine." *Rpf* homologues appear to be confined to high-GC gram-positive organisms and it is particularly noteworthy that the *Mycobacterium tuberculosis* and *M. leprae* genomes both encode multiple homologues (80).

The decline or death phase of batch cultures is a highly variable phenomenon depending on the organism, strain and medium used. Classically the total cell number in the culture is maintained while the CFU count declines. It should be apparent from the foregoing that the stationary phase is only stationary with respect to total cell counts and net biomass. Moreover, the properties of the culture population become less well defined with increasing time after the end of exponential phase and little of substance can be said about the molecular events that occur after the first few days of this period. However, the recent trend to question whether cells that do not produce colonies on the standard culture medium for the organism concerned (e.g., those developing during the decline phase) may, nonetheless, be considered "viable" makes discussion of this topic more appropriate for the final section of this chapter.

Exit from Dormant or Stationary Cellular States and Re-entry into Growth

Static cells probably do not re-enter the growth cycle simply by reversing the process by which they entered stasis (cf. Fig. 1.1). This is clearly so for spores where germination is clearly not the opposite of sporulation; a comparable process is suggested for other forms of dormancy and stasis. Germination has been demonstrated to depend on one or more specific

germination signals, and whether this is so for other forms of static cells is not known (79). Nonetheless, it does seem clear that requirements for initiating growth are somewhat distinct from those necessary for its maintenance.

Defining the nutrients and signals and other conditions necessary for initiating growth is of considerable medical importance. Not only are there several diseases, notably tuberculosis, that have "latent" phases in which it is thought that the pathogen may itself be dormant (4, 39), but also the reliable determination of the presence of organisms in clinical and environmental samples by culture remains absolutely central to patient management and public health monitoring (5). In the former case a complete knowledge of those factors that activate and deactivate growth could enable us to recognise why latent disease reactivates; moreover, we might be able to specifically activate dormant cells to make them susceptible to standard chemotherapy. Regarding cultivation, only when we have a comprehensive understanding of those factors necessary for organisms to initiate growth can we be confident that culture-based detection is at its most sensitive.

Apart from germination, we really know very little about the process of transition from stasis to growth. The discovery of Rpf provides exciting opportunities to study one example of this process particularly because a key assayable effect of this molecule is a reduction in the lag phase. Kaprelyants and Kell have reviewed the evidence relating to other molecules that have effects comparable to Rpf (48). Growth stimulatory molecules affecting both Gram-positive and Gram-negative organisms have been identified with varying degrees of certainty.

It seems likely that signalling molecules play a significant role in growth regulation in at least some species in some environments. It has even been suggested that growth of some species may be completely dependent on molecules like Rpf (48). While this is an interesting proposal, demonstration of Rpf dependency requires very specific environmental conditions and the natural physiological roles for the molecule and its homologues are far from being understood. Whatever these may be, the significance of signalling in bacterial growth cannot be ignored; in particular the possibility that growth is a "social," "communal" or "quorum-dependent" process in bacteria is now firmly on the agenda (see Chapter 4).

CULTURABILITY AND VIABILITY

Molecular analyses have allowed us to test for the presence of specific organisms without demonstrating their capacity to multiply. Such developments challenge us to review the value of culture as a means both of detecting